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## ORIGINAL ARTICLE

## A growth hormone receptor SNP promotes lung cancer by impairment of SOCS2-mediated degradation

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Both humans and mice lacking functional growth hormone (GH) receptors are known to be resistant to cancer. Further, autocrine GH has been reported to act as a cancer promoter. Here we present the first example of a variant of the GH receptor (GHR) associated with cancer promotion, in this case lung cancer. We show that the GHRP495T variant located in the receptor intracellular domain is able to prolong the GH signal *in vitro* using stably expressing mouse pro-B-cell and human lung cell lines. This is relevant because GH secretion is pulsatile, and extending the signal duration makes it resemble autocrine GH action. Signal duration for the activated GHR is primarily controlled by suppressor of cytokine signalling 2 (SOCS2), the substrate recognition component of the E3 protein ligase responsible for ubiquitinylation and degradation of the GHR. SOCS2 is induced by a GH pulse and we show that SOCS2 binding to the GHR is impaired by a threonine substitution at Pro 495. This results in decreased internalisation and degradation of the receptor evident in TIRF microscopy and by measurement of mature (surface) receptor expression. Mutational analysis showed that the residue at position 495 impairs SOCS2 binding only when a threonine is present, consistent with interference with the adjacent Thr494. The latter is key for SOCS2 binding, together with nearby Tyr487, which must be phosphorylated for SOCS2 binding. We also undertook nuclear magnetic resonance spectroscopy approach for structural comparison of the SOCS2 binding scaffold Ile455-Ser588, and concluded that this single substitution has altered the structure of the SOCS2 binding site. Importantly, we find that lung BEAS-2B cells expressing GHRP495T display increased expression of transcripts associated with tumour proliferation, epithelial–mesenchymal transition and metastases ( *Twist1*,  *Snai2*,  *Egfr*,  *Myc* and  *Ccnd1*) at 2 h after a GH pulse. This is consistent with prolonged GH signalling acting to promote cancer progression in lung cancer.

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## INTRODUCTION

Considerable evidence supports a role for the growth hormone (GH)–insulin-like growth factor-1 (IGF-1) axis in cancer incidence and progression.<sup>1</sup> This includes epidemiological studies linking elevated circulating IGF-1 to increased colorectal, breast, prostate and lung cancer incidences.<sup>2–4</sup> Conversely, an absence of cancer has been reported in humans harbouring a GH receptor (GHR) mutation resulting in IGF-1 deficiency.<sup>5</sup> Moreover, rodent models lacking GH or its cognate receptor (GHR) are strikingly resistant to the induction and severity of a wide range of cancers,<sup>6</sup> and treatment with pegvisomant (GHR antagonist) can slow tumour progression.<sup>7</sup> Although expression of *GHR* is elevated in many cancers such as primary ductal invasive breast cancer,<sup>2</sup> autocrine GH is present in several cancer types and predicts a worse outcome of mammary and endometrial cancers.<sup>8</sup> Forced expression of autocrine GH has been shown to induce cell transformation.<sup>9</sup>

Two independent studies showed that a single-nucleotide polymorphism (SNP) in *GHR* (C to A on *GHR*1526; rs6183) resulting in an amino-acid change at position 495 from proline to threonine (P495T) was associated with lung cancer. A genome-wide

association study (GWAS) identified *GHRP495T* with a striking odds ratio (OR) of 12.98 in a Caucasian population of smokers (94%).<sup>10</sup> The other report, a comprehensive study in a Han Chinese population, showed that *GHRP495T* was associated with lung cancer with an OR of 2.04.<sup>11</sup> This latter study showed that *GHRP495T* was strongly associated with lung cancer in the sub-populations with higher risk for lung cancer (males, still-smokers, and the sub-population with familial history of cancer), was significantly associated with small cell and squamous cell lung cancer, and was proposed to carry a familial risk for these cancers. A recent SNP analysis of non-small cell lung cancer (NSCLC) in a population-based study of women comparing Caucasians and African-Americans identified three other SNPs in linkage disequilibrium with *GHRP495T* that associated with a 50% increased cancer risk and indicated that the disease marker could well be *GHRP495T*. This study also confirmed the association of SNPs in this region of *GHR* with smoking.<sup>12</sup>

There is currently no functional role attributed to GHR P495, however, Y487 is phosphorylated (pY487) upon GHR activation and this forms a known binding site for signal transducer and activator of transcription 5 (STAT5) and for suppressor of cytokine

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signalling 2 (SOCS2),<sup>13</sup> a ubiquitin ligase negatively regulating *GHR* expression and downstream JAK2/STAT5 signalling.<sup>14</sup> SOCS protein levels are constitutively low, but their expression increases rapidly following cytokine stimulation.<sup>15</sup> GH induces the expression of several SOCS proteins *in vitro*<sup>16,17</sup> and *in vivo*.<sup>18</sup> Generally, SOCS1, SOCS3 and cytokine-inducible SH2-containing protein (CISH) expression is rapidly induced following GH stimulation, but is short-lived, whereas SOCS2 expression increases steadily with time.<sup>19</sup> There is evidence that binding to pY487 and pY595 is required for SOCS2-mediated inhibition on GH action.<sup>20</sup> As both tyrosines are known STAT5 binding sites, SOCS2 can competitively inhibit STAT5 receptor binding.<sup>21,22</sup> Recently, pY487 rather than pY595 was assigned the key role for regulation by SOCS2, and it was further shown that SOCS2 regulates cellular GHR levels through ubiquitinylation-dependent proteasomal degradation.<sup>13</sup> Importantly, of SOCS proteins, only SOCS2 knockout mice show a significant increase in size (~40%) compared with wild-type mice.<sup>23</sup> This supports the key role of SOCS2 in GHR signalling. Accordingly, to elucidate the role of the *GHRP495T* in lung cancer promotion, we have investigated the effect of the P495T substitution on GHR structure, signalling and regulation by SOCS2 activity.

## RESULTS

### GHR expression in normal and cancerous lung tissue

Analysis of a gene expression microarray GEO data set for *GHR* transcript levels comparing 18 squamous cell carcinoma (SCC) and 40 lung adenocarcinoma tissues (GSE10245)<sup>24</sup> showed higher *GHR* levels in SCC than in adenocarcinoma (Figure 1a). This is consistent with the *GHRP495T* variant being significantly associated with small cell and SCC, but not with adenocarcinoma. To further investigate relative expression levels in a NSCLC cohort, bioinformatics analysis (GSE19804)<sup>25</sup> of transcript levels in clinical samples from 60 normal lung tissues and 60 NSCLC tumours from non-smoking females was performed. There was significantly higher *GHR* and *EGFR* expression in NSCLC (Figures 1b and c), with a relative decrease in *SOCS2* and *SOCS3* levels in NSCLC (Figures 1d and e) but no evident difference in *SOCS1*, *CISH*, and *GH2* transcript levels (Supplementary Figure 1).

### The *GHRP495T* enhances GH-mediated signalling

We first compared proliferative signalling in Ba/F3 cells transduced to express human *GHRP495T* or wild-type (WT) *GHR* in Ba/F3, with surface receptor levels matched by fluorescence-activated cell sorting using an N-terminal HA-tag. Although no constitutive activation was evident in the *GHRP495T*, cell proliferation was significantly increased in response to 4.5 nM human GH (hGH) (Figure 2a). GH secretion is highly pulsatile in males, with somewhat lower amplitude pulses in females,<sup>26–28</sup> thus starved cells were stimulated with 2.3 nM hGH for 15 min, then washed, and a cell fraction harvested at subsequent time intervals. At 90–150 min after the GH pulse, phosphorylated STAT5, the major effector of GH-induced proliferation in these cells, was significantly increased for *GHRP495T* relative to WT *GHR* cells (normalised to  $\beta$ -TUBULIN) and relative to GHR levels (Figures 2b and c). When we investigated multiple transduced and selected cell lines based on fluorescence-activated cell sorting for equal surface GHR, each time the *GHRP495T* gave elevated total GHR levels compared with WT *GHR* in the absence of GH (Figure 2b). We attributed this to the reduced degradation/turnover of the *GHRP495T* within the cell compared with WT *GHR* cells. In the absence of any exogenous hGH, no STAT5 activation was evident as murine GH (poorly expressed in Ba/F3 cells) and bovine GH (small amounts present in the serum) do not activate human GHR.<sup>29,30</sup>

We next examined GH signalling in a non-tumourigenic human bronchial epithelial lung cell line (BEAS-2B) transduced to express

matched levels of WT *GHR* or *GHRP495T*. Proliferation was enhanced in *GHRP495T* cells in response to 2.3 nM and 4.5 nM hGH (Figure 3a) based on BrdU incorporation. Signalling in response to a GH pulse showed that AKT signalling (measured by AKT pT308) increased significantly in *GHRP495T* cells from 45 min after the GH pulse (Figures 3b and c). AKT-T308 phosphorylation is a strong prognostic indicator for NSCLC.<sup>31</sup> Similarly, phospho-STAT3 levels (pY705) were significantly elevated 45–60 min after the GH pulse (Figures 3d and e). Activated STAT3 is an important oncogenic factor during carcinogenesis and metastasis of SCLC and squamous cell lung carcinoma, and correlates with clinical stage, prognosis, and lymph node metastasis,<sup>32</sup> as well as smoking history.<sup>33</sup>

### The *GHRP495T* impairs SOCS2 binding

No difference was found between the ability of HEK293 cells expressing WT *GHR* or *GHRP495T* to induce *SOCS2* transcript upon GH stimulation (Figure 4a). However, co-immunoprecipitation (co-IP) analysis of SOCS2 with WT *GHR* or *GHRP495T* demonstrated that SOCS2 binding to the activated *GHRP495T* receptor was markedly impaired (Figures 4b and c). No significant interaction between CISH and WT *GHR* was observed (Figure 4d), consistent with the *in vivo* situation where in hepatic tissue derived from WT mice we observed continuous turnover of SOCS2, but not CISH (Supplementary Figure 2). As expected, a decline in *socs2* and *cish* transcript levels was observed in *ghr*<sup>-/-</sup> mice because of lack of GHR-activated STAT5. In contrast, SOCS2 protein levels were high, whereas no difference in CISH protein level was observed between *ghr*<sup>-/-</sup> and WT mice. This result is consistent with impaired turnover of SOCS2 because of the lack of GHR-mediated proteolysis, whereas CISH was unaffected because of its lack of direct interaction with GHR.

### Mutational analysis of SOCS2 binding to GHR

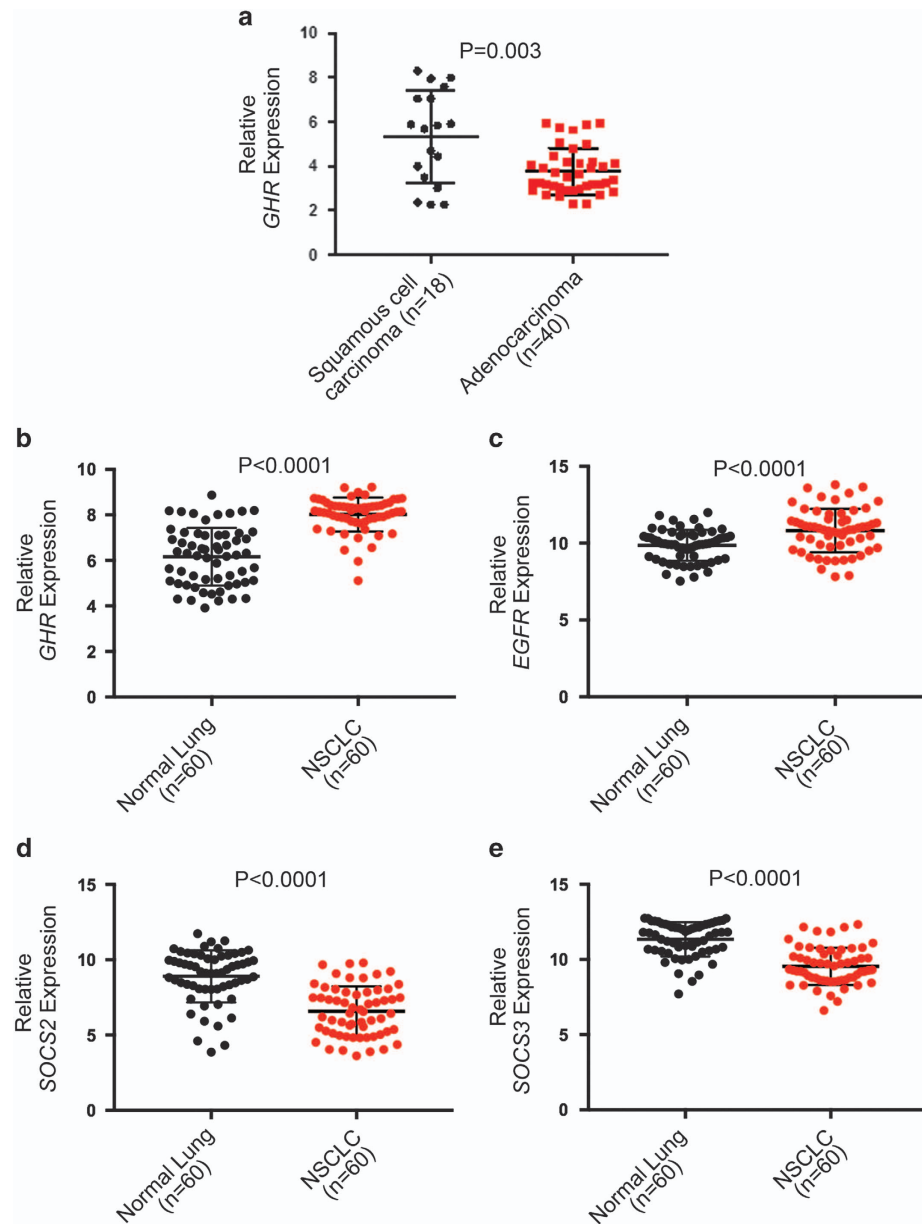
To investigate the conservation of the GHR P495 and the surrounding residues, we performed a Clustal Omega alignment with other species and identified P495 and residues in close proximity to be highly conserved (Figure 5a). Mutational analysis of these conserved residues revealed that alanine or lysine substitution of P495 did not significantly alter SOCS2 binding to GHR, however, substitution of the adjacent T494 with alanine strongly impaired its binding (Figures 5b and c).

### *GHRP495T* causes structural changes in the intracellular domain

We used nuclear magnetic resonance spectroscopy to define the structural propensity of the WT *GHR* and *GHRP495T* using the intracellular region of the receptor spanning I455–S588. We recently reported that the cytoplasmic domain is intrinsically disordered, but with eight 10–30% transiently populated helices (THs).<sup>34</sup> Compared with other amino acids, proline gives conformational rigidity. P495 resides between TH5.2 and TH6, and has no helical propensity. Mutation to threonine induced a new transient structure, TH5.3 with effects also on TH5.2 (Figure 5d). This change in the structural ensemble could bring T495 closer to Y487 and within proximity of the key arginine residues of SOCS2 causing steric hindrance/interference. Moreover, increased helicity around Y487 may put I484 and V490 (i-3, i +3), both SOCS2 specificity residues, in an orientation facing away from Y487, which may add to reduce binding efficiency to SOCS2 that relies on intrinsic disorder and formation of extended structure.<sup>35,36</sup>

### *GHRP495T* degradation is impaired

We studied the effect of *GHRP495T* on receptor turnover with and without co-transfection of SOCS2. HEK293 cells were pre-treated with Brefeldin A (BFA) (1  $\mu$ g/ml) to block incorporation of new



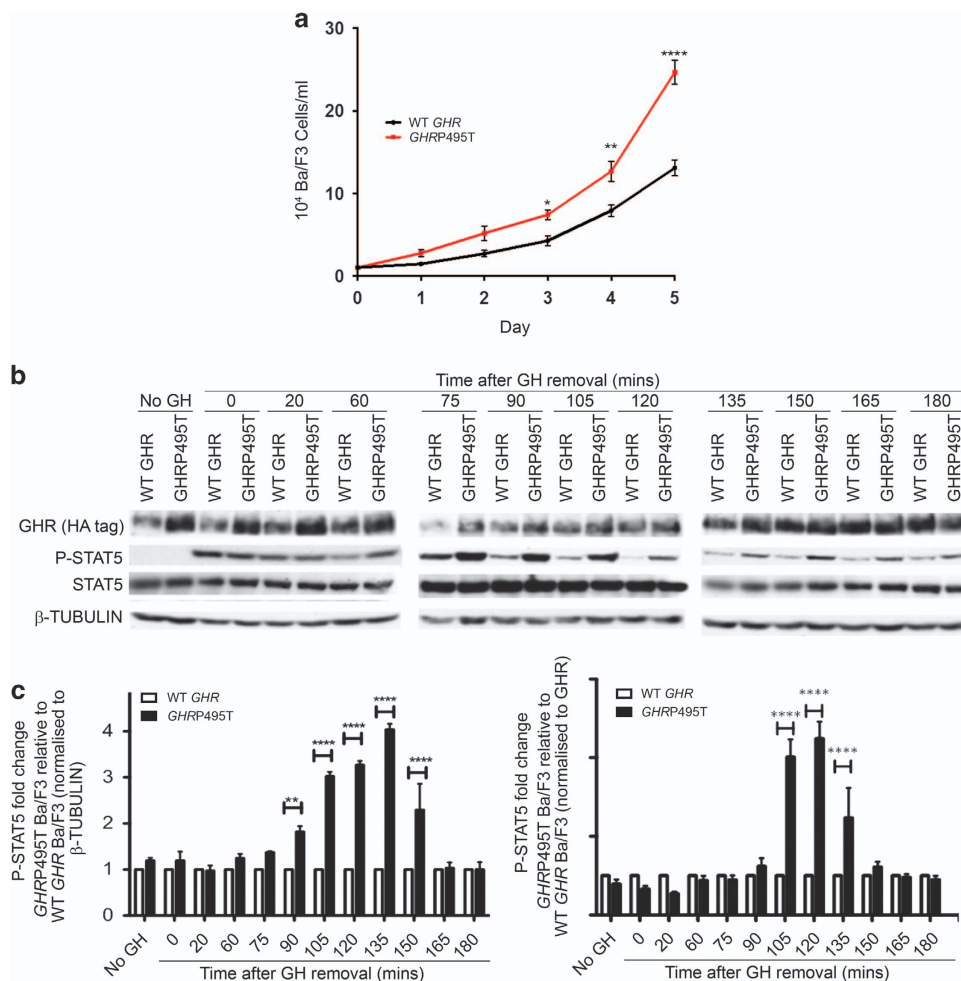
**Figure 1.** *GHR* expression in normal and cancerous lung tissue. (a) *GHR* levels in clinical samples representing 18 SCC and 40 lung adenocarcinoma analysed from microarray data (GSE10245) retrieved from Gene Expression Omnibus (GEO).<sup>24</sup> A significant correlation was determined at  $P = 0.003$ . Gene expression analysis of four genes (b) *GHR*, (c) *EGFR*, (d) *SOCS2* and (e) *SOCS3* in 60 clinical samples of normal lung tissue and NSCLC from a non-smoking female cohort in accordance with microarray data (GSE19804).<sup>25</sup> A significant difference was observed ( $P < 0.0001$ ). The expression levels of other genes (*SOCS1*, *CISH* and *GHR2*) did not differ significantly (Supplementary Figure 1).

receptor into the plasma membrane, followed by a 15-min GH pulse, and followed the rate of disappearance of the mature (upper) GHR band in immunoblots. Degradation of the mature surface GHRP495T was significantly less than WT GHR in response to GH (Figures 6a and b) and this effect was exacerbated by transfection of a *SOCS2* expression plasmid (Figures 6c and d).

A time course assay was performed in BEAS-2B cells with a second dose of GH at 60 and 120 min; time points where the levels of mature GHR were very different between WT GHR and GHRP495T (Figure 3b). Following the second acute GH stimulation, GHRP495T was clearly less prone to degradation than WT GHR (Supplementary Figure 3).

In order to investigate surface GHR, HEK293 cells transduced to stably express WT or GHRP495T each with a C-terminal fusion to GFP were transiently transfected with a *SOCS2*-mCherry

expression plasmid. The presence of both *SOCS2* and GHRP495T at the basal plasma membrane of the GHRP495T-expressing cells was clearly observed by TIRF microscopy, but not for WT GHR where it was largely absent in the presence of *SOCS2*-mCherry (Figure 6e). Quantitation of these observations showed a significant decrease in WT GHR at the basal plasma membrane in the presence of *SOCS2*, but not for the GHRP495T receptor (Figure 6f), whereas *SOCS2* fluorescence showed no difference in its plasma membrane level between WT and GHRP495T (Figure 6g). Co-IP analysis showed no difference in *SOCS2* binding between GFP-tagged and non-GFP-tagged WT GHR (Supplementary Figure 4), demonstrating that *SOCS2* interaction was unaffected by GFP-fusion. When cells were subjected to proteasome inhibitors MG-132 or clasto-Lactacystin  $\beta$ -lactone more mature precursor was seen in the WT GHR relative to



**Figure 2.** *GHRP495T* increases proliferation owing to enhanced GH-mediated signalling in pre-B cells. **(a)** Proliferation assay in Ba/F3 cells transduced with WT *GHR* or *GHRP495T* seeded at  $1 \times 10^4$  cells/ml in growth medium (devoid of IL-3) containing GH (4.5 nM). Cells were counted daily by Trypan blue exclusion using haemocytometer over a 5-day period. Data presented as mean  $\pm$  s.e.m. analysed by two-way ANOVA (\*\*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$ ) and representative of three independent experiments performed in duplicate and confirmed in three independently transduced cell lines. **(b)** Time course analysis of GHR-mediated signalling in Ba/F3 cells transduced with WT *GHR* or *GHRP495T*. Serum-starved cells were subjected to an acute GH (2.3 nM) dose for 15 min and harvested at the indicated time points. Cell lysates were immunoblotted for P-STAT5 (Tyr694/699), total STAT5 and GHR (HA-tag) across all time points and the expression was compared with  $\beta$ -TUBULIN (loading control). **(c)** P-STAT5 (Tyr694/699) signal intensity represented as fold change with respect to WT *GHR* at all time points and normalised to  $\beta$ -TUBULIN and total GHR (HA-tag) levels. Data presented as mean  $\pm$  s.e.m. analysed by two-way ANOVA (\*\*\*\* $P < 0.0001$ , \*\* $P < 0.01$ ) and representative of three independent experiments confirmed in cell lines fluorescence-activated cell sorted (FACS) for similar surface GHR expression.

*GHRP495T*-expressing cells, supporting the more rapid proteasomal degradation of the WT receptor, in accordance with its intact degron and SOCS-binding site (Figure 6h and Supplementary Figure 5). In addition, in HEK293 transduced cells expressing WT or *GHRP495T* we observed GHR remnants at approximately 60 and 43kDa only in the WT *GHR* lysates in the absence of hormone, which may be the result of hormone independent endocytosis/turnover, possibly through the phospho-degron.<sup>37</sup>

The *GHRP495T* increases transcripts associated with tumour progression

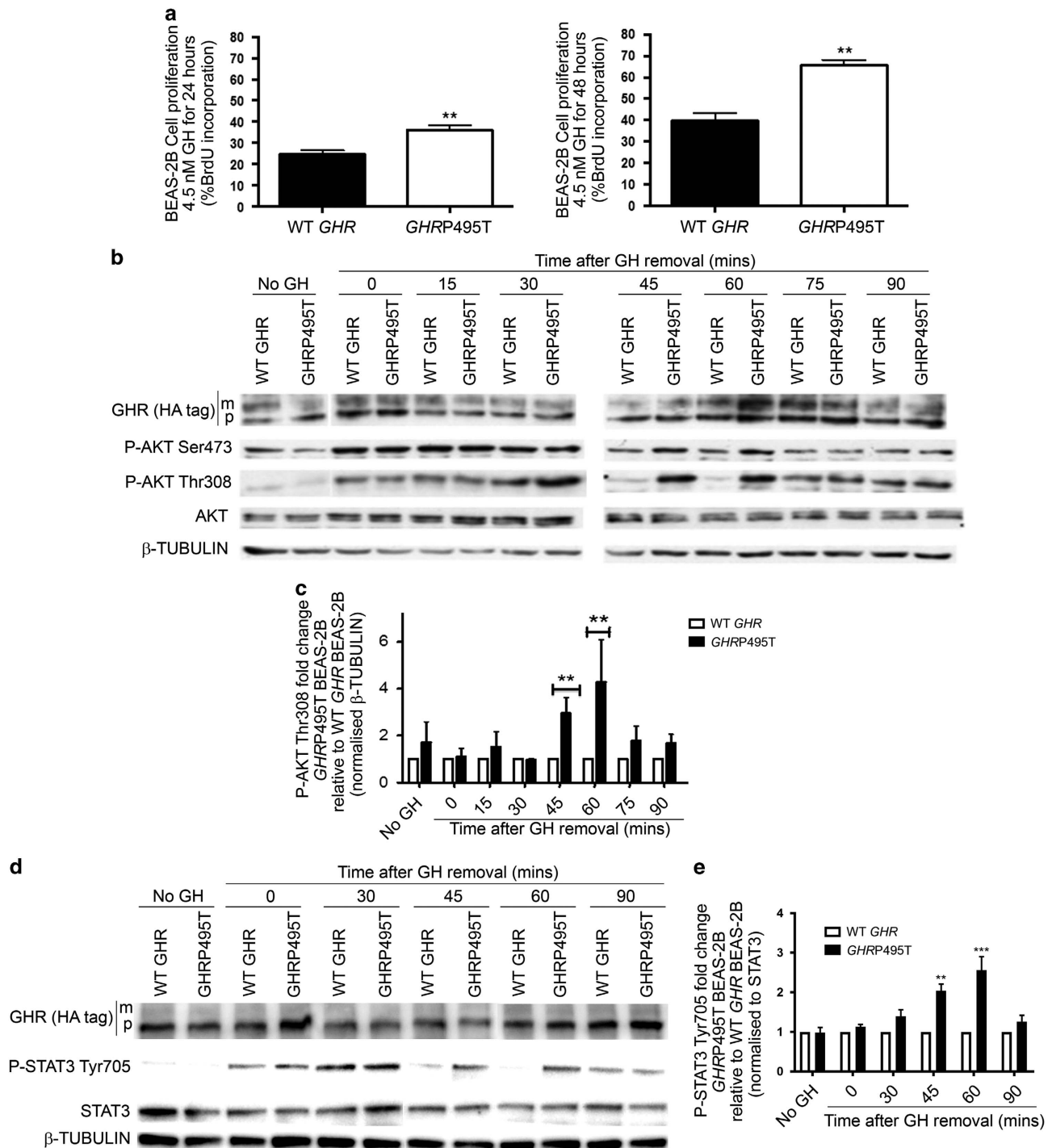
Real-time PCR analysis of BEAS-2B cells transduced to express WT *GHR* or *GHRP495T* following a single 15-min pulse of GH showed significant increases for *FOS* and *EGR1*, known GH targets<sup>38</sup> at 60-min post-stimulation (Figure 7a). Moreover, at 120 min after the initial GH stimulation, a subset of genes associated with proliferation, epithelial–mesenchymal transition and cancer metastasis ( *Twist1*,  *SNAI2*,  *EGFR*,  *MYC* and  *CCND1*) was significantly

upregulated only in *GHRP495T*-expressing cells compared with WT *GHR* (Figure 7b). Elevated levels of most of these genes are known prognostic predictors in patients with NSCLC.<sup>39,40</sup> It is also relevant that GHR signalling strongly induced the expression of epidermal growth factor receptor ( *EGFR*)<sup>41</sup> as observed in BEAS-2B cells (Figure 7b and Supplementary Figure 6).

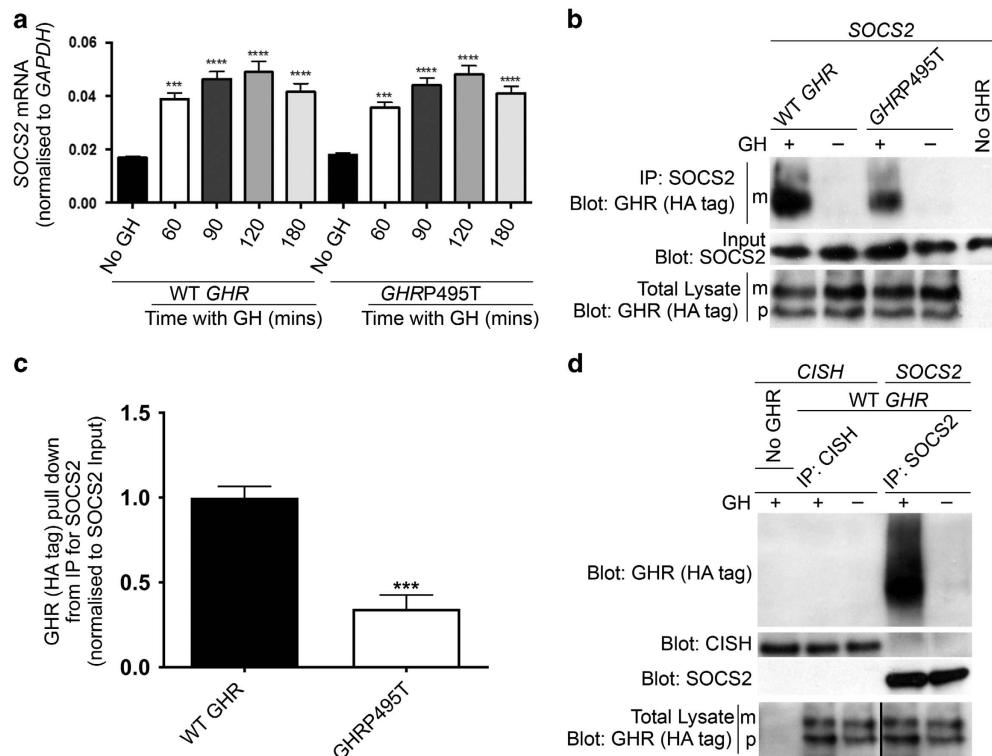
## DISCUSSION

Two independent studies of different ethnic groups identified a SNP in *GHR* that resulted in the residue change P495T and increased risk of lung cancer.<sup>10,11</sup> We investigated the molecular basis for how this change may contribute to increased incidence of lung cancer. Ba/F3 cells expressing *GHRP495T* showed a prolonged STAT5 activation following a GH pulse with a higher residual receptor expression than WT *GHR* transduced cells and a significant increase in active STAT5 when normalised to receptor levels. STAT5 activation has been reported in cancers of the breast, prostate, lung and leukaemia.<sup>1</sup> Constitutively active STAT5





**Figure 3.** GHRP495T enhances GH-mediated signalling in a normal lung cell line. (a) BrdU incorporation assay in BEAS-2B cells transduced with WT GHR and GHRP495T grown on coverslips in growth medium supplemented with GH (4.5 nM) over 24 and 48 h. Cells were treated with 20  $\mu$ M BrdU and subjected to immunofluorescence. Graphs represent percentage of BrdU-positive cells relative to total number of cells (DAPI) counted in random fields of view. Data presented as mean  $\pm$  s.e.m. analysed by Student's *t*-test ( $***P < 0.01$ ) and representative of three independent experiments performed in two independently transduced lines. (b) Time course analysis of GHR-mediated signalling in BEAS-2B cells transduced with WT GHR and GHRP495T. Serum-starved cells were subjected to acute GH (2.3 nM) dose for 15 min and harvested at the indicated time points. Cell lysates were immunoblotted for P-AKT (Ser473 and Thr308), total AKT and GHR (HA-tag) (mature (m) receptor and precursor (p) receptor) across all the time points and compared with  $\beta$ -TUBULIN (loading control). (c) P-AKT (Thr308) signal intensity is represented as fold change with respect to WT GHR at all time points and normalised to  $\beta$ -TUBULIN levels. (d) BEAS-2B lysates as above immunoblotted against P-STAT3 (Tyr705) and GHR (HA-tag) (mature (m) receptor and precursor (p) receptor) compared with GAPDH (loading control). (e) P-STAT3 (Tyr705) signal intensity is represented as fold change with respect to WT GHR at all time points and normalised to total STAT3 levels. Data presented as mean  $\pm$  s.e.m. analysed by two-way ANOVA ( $***P < 0.001$ ,  $**P < 0.01$ ,  $*P < 0.05$ ) and representative of three independent experiments confirmed in three separate lines generated by independent transductions.

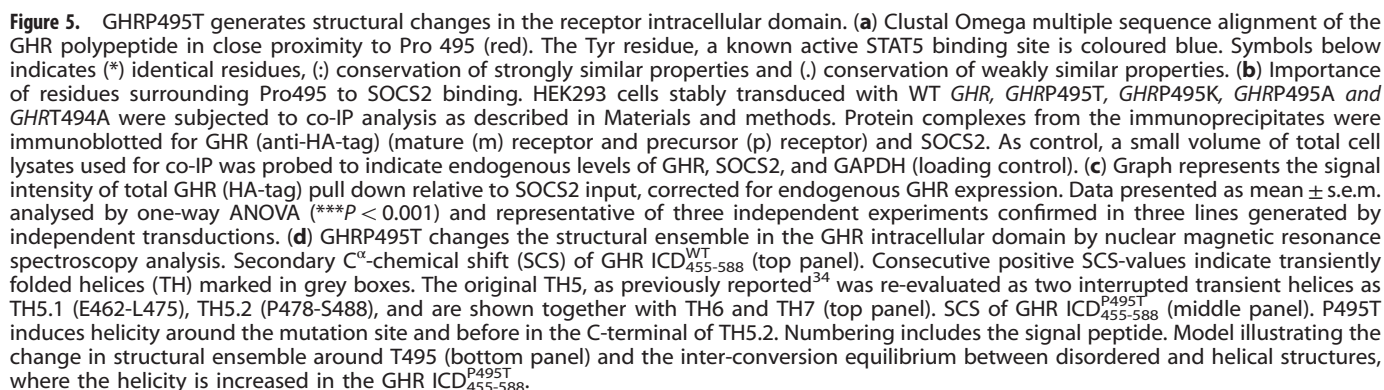


**Figure 4.** GHRP495T impairs SOCS2 binding to GHR. **(a)** No difference in *SOCS2* transcript induction between WT *GHR* and *GHRP495T*. HEK293 cells transduced with WT *GHR* and *GHRP495T* were maintained in serum-starved media with sustained GH (2.3 nM) then RNA was extracted at indicated time points. *SOCS2* levels were determined by real-time PCR and normalised to *GAPDH* reference gene. Data presented as mean  $\pm$  s.e.m. analysed by one-way ANOVA (\*\*\*\* $P$  < 0.0001, \*\*\* $P$  < 0.001). Representative of three independent experiments confirmed in three separate lines generated by independent transductions. **(b)** HEK293 cells stably expressing WT *GHR* or *GHRP495T*, and parental cells transfected with *SOCS2* expression plasmid for 24 h and serum-starved overnight before 2.3 nM GH (+) or vehicle (-) stimulation for 15 min. Lysates were harvested and co-IP with *SOCS2* antibody as described in Materials and methods. Protein complex from the immunoprecipitates were immunoblotted (IB) using anti-HA antibody for GHR (mature (m) receptor and precursor (p) receptor) and anti-*SOCS2* (input). As control, parental cell line with no transduced GHR was used and a small volume of total cell lysates used for co-IP was probed for GHR levels (HA-tag) to indicate GHR levels. **(c)** Graph represents the signal intensity of total GHR (HA-tag) pull down in *GHRP495T* relative to WT *GHR* relative to *SOCS2* input, corrected for endogenous GHR expression. Data presented as mean  $\pm$  s.e.m. analysed by Student's *t*-test (\*\*\* $P$  < 0.001) and representative of nine independent experiments confirmed in three independently transduced cell lines. **(d)** *CISH* protein does not interact directly with GHR as compared with *SOCS2*. HEK293 cells stably expressing WT *GHR* co-transfected with *CISH* or *SOCS2* expression plasmids for 24 h and serum-starved overnight before 2.3 nM GH (+) or vehicle (-) stimulation for 15 min. Lysates were harvested and co-IP with *SOCS2* and *CISH* antibodies simultaneously. Protein complex from the immunoprecipitates was immunoblotted using anti-HA antibody (for GHR) and anti-*SOCS2* and anti-*CISH* antibodies. As a control, parental cell line with no transduced GHR, but transfected with *CISH* was used and a small volume of total cell lysates used for co-IP was probed for GHR levels (HA-tag) to indicate endogenous GHR levels (see Supplementary Figure 2).

transgenic mice show a 22% increased incidence of developing mammary tumours.<sup>42</sup> STAT5 has been shown to induce production of reactive oxygen species and DNA damage, which are important for cancer initiation.<sup>43</sup> In BEAS-2B cells, expressing either WT *GHR* or *GHRP495T*, weak STAT5 activation was evident but not significantly different following a GH pulse (data not shown). However, a significant increase in active AKT (pT308) and STAT3 levels compared with WT was observed, together with sustained surface GHR. Importantly, the increase in GHR levels was exacerbated following a second GH pulse indicating that GH-induced degradation is impaired in *GHRP495T*, in line with impairment of a phospho-degron involving T494-P495 as described below. It has been shown that pT308 active AKT is a better prognostic indicator for NSCLC than phosphorylated-S473.<sup>31</sup>

HEK293 cells stably expressing WT *GHR* or *GHRP495T* and overexpressing *SOCS2* showed significantly decreased *SOCS2* binding to *GHRP495T* after GH stimulation. This may stem from the steric hindrance combined with a potential altered phosphorylation pattern, and can explain the increase in STAT5 and AKT

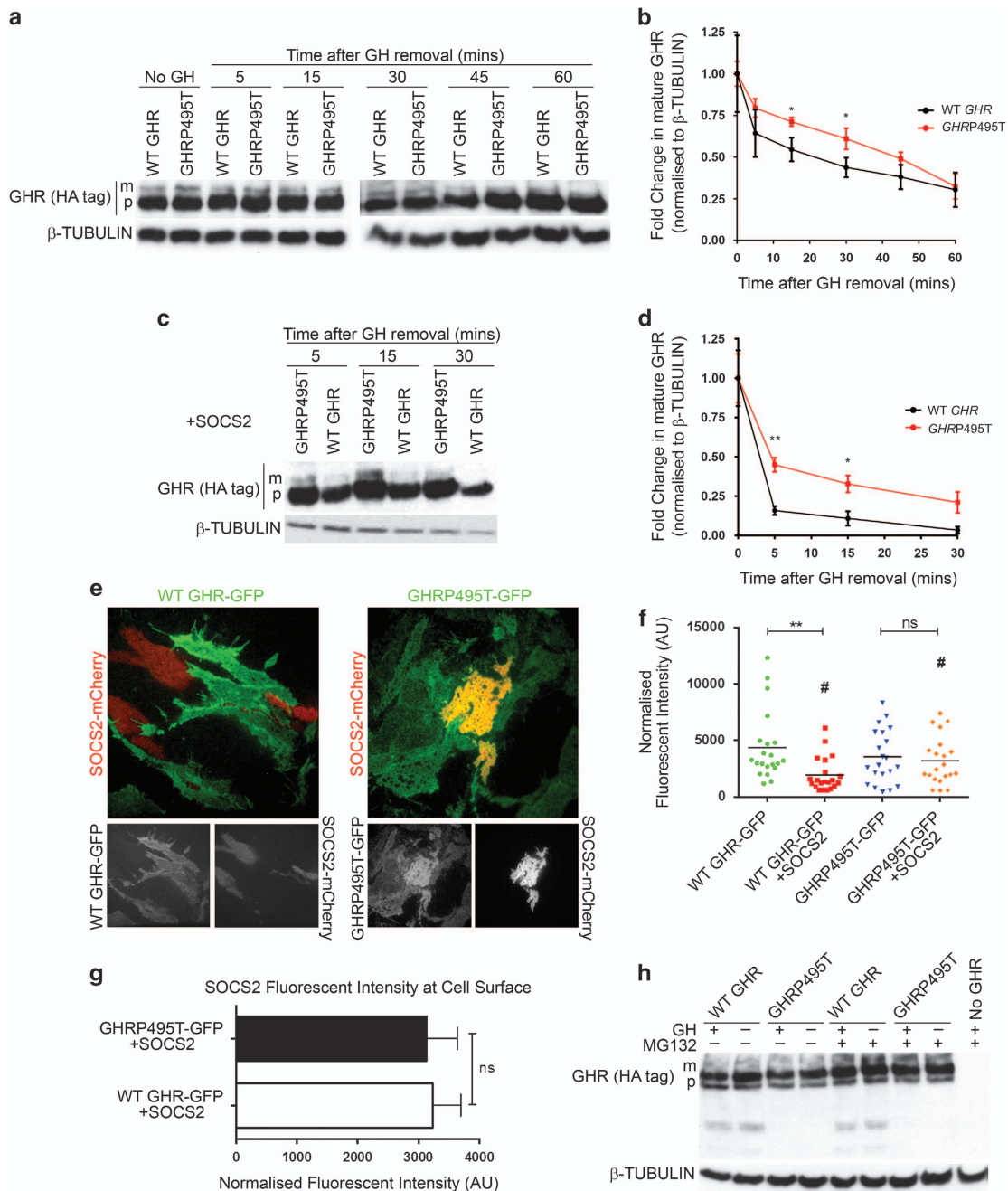
signals observed in the variant as a result of delayed degradation. Both *CISH* and *SOCS2* are expressed in wide range of tissues with highest expression in lung, liver, heart and skeletal muscle.<sup>44</sup> We investigated the interaction of GHR with *CISH* as this is predicted to bind to GHR-pY487 and -pY595. We could not detect any direct interaction between GHR with *CISH*, despite being reported in rat adipocytes.<sup>45</sup> *CISH* may depend on another protein for binding to GHR potentially absent in HEK293 cells, or the interaction between the disordered GHR and *CISH* may be too transient for co-IP detection or require other post-translational modifications. Our data from hepatic tissues from WT and *ghr*<sup>-/-</sup> mice support that *SOCS2* binds and regulates degradation of GHR, whereas *CISH* does not. This data showed that although *socs2* and *cish* transcript levels are dependent on GHR function, *CISH* protein did not change between WT and *ghr*<sup>-/-</sup>, but *SOCS2* accumulated to high protein levels in the absence of GHR implying that *SOCS2* binding to GHR led to its degradation, potentially exploiting the degron as suggested below. *SOCS2* has been shown to have a major role in growth, as *socs2* is the only *socs* member where knockout mice are giants, whereas *cish*<sup>-/-</sup> are not reported to have any growth



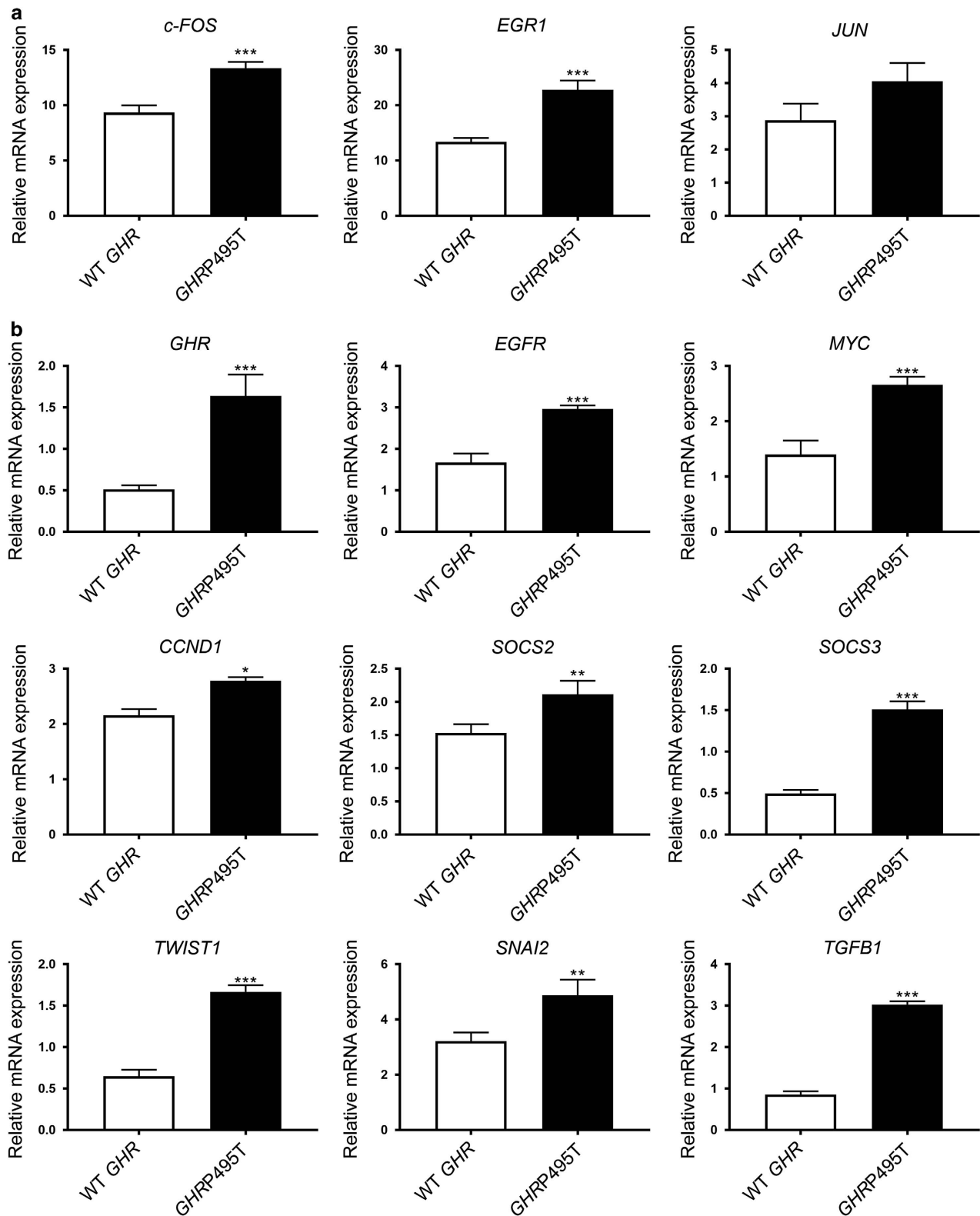
To confirm that increased signalling in GHRP495T cells is a surface phenomenon, we pre-treated cells with BFA before GH stimulation and found a significant decrease in mature WT receptor on the cell surface compared with GHRP495T and the increase in GHRP495T level was maintained throughout the time course analysis. The rate of WT GHR degradation was considerably

We investigated the structural changes induced by P495T in GHR using nuclear magnetic resonance analysis of a portion of the GHR intracellular region. Comparison between WT GHR and GHRP495T indicated an altered structural ensemble and the region around P495T more helical. This potentially affects the binding region for kinases, SOCS2, and others such as FBW7, and is evidently sufficient to cause a phenotypic effect in *GHRP495T* heterozygous patients, also significantly more susceptible to lung





**Figure 6.** GHRP495T degradation is impaired. **(a)** Effect of BFA treatment on WT GHR and GHRP495T. Time course analysis on HEK293 cells transfected with WT *GHR* and *GHRP495T* subjected to 2.3 nM GH stimulation for 15 min in the presence of BFA and harvested at indicated time points. Immunoblot demonstrating GHR (HA-tag) levels (mature (m) receptor and precursor (p) receptor) and β-TUBULIN (loading control). **(b)** Graph indicating fold change in mature GHR levels normalised to β-TUBULIN relative to '0 min' time point after GH removal. **(c)** Immunoblot indicating GHR (HA-tag) following BFA treatment, as above **(a)** in the presence of SOCS2. **(d)** Graph indicating fold change in mature GHR levels in the presence of SOCS2, normalised to β-TUBULIN relative to '0 min' time point after GH removal. **(b, d)** Data presented as mean ± s.e.m. analysed by two-way ANOVA (\*\**P* < 0.01, \**P* < 0.05) and representative of at least three independent experiments confirmed in two independently transfected lines (see Supplementary Figure 3); **(e)** GHRP495T is less amenable to degradation owing to SOCS2 as evident from TIRF microscopy (allows detection of fluorescent proteins only at, or near the cell membrane) images of HEK293 cells transfected with WT GHR-GFP and GHRP495T-GFP transfected with SOCS2-mCherry in the absence of exogenous GH. Colocalisation (yellow) of SOCS2 (red) and receptor (green) was more pronounced in GHRP495T (See Supplementary Figure 4). Separate channel images shown below. **(f)** GHR levels on cell surface of HEK293 cells expressed as normalised fluorescent intensity in the presence or absence of SOCS2. Data presented as mean ± s.e.m. from 30 cells per condition across two independently transfected cell lines and analysed by one-way ANOVA (\*\**P* < 0.01) relative to WT GHR-GFP no SOCS2. Student's *t*-test (\**P* < 0.05) between WT GHR-GFP + SOCS2 and GHRP495T-GFP + SOCS2. **(g)** SOCS2 levels at the cell surface expressed as normalised fluorescent intensity for the cells analysed above. Data presented as mean ± s.e.m. from 30 cells per condition from three independent experiments across two independently transfected lines and analysed by Student's *t*-test (ns, not significant). **(h)** HEK293 cells transfected with WT *GHR* and *GHRP495T* treated with proteasomal inhibitor MG-132 (10 μM) for 2 h before GH (2.3 nM) addition for 15 min. Immunoblot indicating GHR (HA-tag) levels with mature and precursor receptor and two remnant bands observed at ~60 and ~43 kDa only in WT GHR lysates and undetectable in GHRP495T (see Supplementary Figure 5). Blot representative of four independent experiments.



**Figure 7.** GHRP495T increases transcripts associated with tumour progression. BEAS-2B cells transduced with WT *GHR* and GHRP495T stimulated with GH (2.3 nM) for 15 min, then RNA was harvested at 60 (a) and 120 min (b) post-initial treatment. (a) Transcript levels of early GH-response genes *c-FOS*, *EGR1*, *JUN* at 60 min in WT *GHR* and GHRP495T cells normalised to *B2M*. (b) Transcript levels of genes associated with epithelial–mesenchymal transition (EMT) (*TWIST1*, *SNAI2* and *TGFB1*), proliferation (*CCND1*, *MYC*), and elevated GHR signalling (*GHR*, *EGFR*, *SOCS2* and *SOCS3*) at 120 min in WT *GHR* and GHRP495T cells normalised to *B2M* (see Supplementary Figure 6). Data presented as mean  $\pm$  s.e.m. analysed by one-way ANOVA (\*\*\* $P$  < 0.001, \*\* $P$  < 0.01, \* $P$  < 0.05) and representative of three independent experiments confirmed in three independently transduced lines.

cancer.<sup>11</sup> Prolactin receptor degradation is regulated by serine phosphorylation where glycogen synthase kinase-3 $\beta$  phosphorylation of prolactin receptor S349 is required for recognition by  $\beta$ TrCP and subsequent ubiquitylation and degradation.<sup>50,51</sup> Studies on GHR have only focused on tyrosine phosphorylation, however, serine and threonine residues comprise 9% and 6%, respectively, of GHR, whereas tyrosine makes up only 2.8%. It is currently unknown if any of these serine and threonine residues are phosphorylated or what kinases may be involved. The eukaryotic linear motif (ELM) database ([www.elm.eu.org](http://www.elm.eu.org)) predicts T494 as a target for glycogen synthase kinase-3, cyclin dependent kinase 1 or mitogen activated protein kinase, as well as T494-P495 being parts of a phospho-degron (TPxxxS) recognised by the ubiquitin ligase FBW7.<sup>52,53</sup> Thus, the sequence properties around P495 constitute a phospho-degron motif. Our co-IP data suggested T494 to be important for SOCS2 binding and it could be speculated that phosphorylation of T494 may facilitate SOCS2 binding to GHR. Therefore, besides the change in structure, the new threonine in GHRP495T may potentially become phosphorylated or hinder the phosphorylation of T494 resulting in further impaired SOCS2 or FBW7 binding; a protein also requiring extended, disordered structure for binding.<sup>52</sup>

It has been reported that overexpressed or constitutively active EGFRs are major drivers in lung cancer as their expression supports survival, proliferation and metastases, hence protection from cytotoxic agents and radiation therapies.<sup>54</sup> In addition, the EGFR-STAT3 signalling axis promotes tumour survival in NSCLC.<sup>55</sup> Given the ability of GH to induce EGFR signalling, together with STAT3 and AKT activation, the pulsatile manner of GH secretion (particularly in males, the more lung cancer susceptible sex<sup>11</sup>), the extension of GH signalling in GHRP495T appears highly relevant to its ability to promote lung cancer progression in individuals particularly those with smoking-induced lung cancers. We expect that the prolonged downstream GHR signalling is acting as a promoter in response to DNA damage caused by the inhaled smoke, particularly the reactive oxygen species component.<sup>56</sup>

This study highlights the first identified cancer associated variant of *GHR* and shows that the P495T variant prolongs signalling in cell lines associated with reduced degradation of the GHR. This reduction can occur in a GH-dependent manner because of decreased SOCS2 binding or in the absence of GH by alteration of the GHR turnover via modulation of a phospho-degron. Future research using an animal model carrying the variant allele is warranted and can shed light on the role of GHRP495T in tumour initiation or promotion. It is currently unknown if GHRP495T polymorphism is associated with other cancer types and if the cancer susceptibility will be tissue specific, which will likely depend on the abundance of signal activators and suppressors. Nevertheless, GHRP495T represents a novel candidate SNP that has not been covered on the SNP arrays of recent GWASs for assessing lung cancer risk, but clearly should be included.

## MATERIALS AND METHODS

### Expression vectors

WT human GHR with an introduced N-terminal haemagglutinin (HA)-tag following the signal peptide was amplified by PCR with *attB* adapted primers from a previously described clone in pCDNA3.1<sup>57</sup> and Gateway cloned into pQCXP CMV/TO DEST<sup>58</sup> (a gift from Eric Campeau; Addgene, Cambridge, MA, USA; #17386) and pMX-GW-IRES-Puro. Primers for generation of the GHR mutations P495T, P495A, P495K and T494A were created essentially as described<sup>59</sup> and cloned into the same destinations vectors as for WT GHR. pMX-GH-IRES-Puro was created from pMX-IRES-GFP<sup>60</sup> by replacing the stuffer fragment with *attR* flanked *ccdB*-Cm<sup>R</sup>, and replacing GFP with PuroR. C-terminal GFP-tagged constructs were created by overlap extension PCR. Primer sequences are available on request. SOCS2 expression plasmid pCMV-SOCS2-Flag was purchased from Origene

(Rockville, MD, USA) and CISH expression plasmid was a kind gift from Dr Patrick Lau (IMB, The University of Queensland).

### Cell culture, transfection analysis and treatments

Ba/F3 cells (kindly provided by Dr Andrew J Hapel, Australian National University, Canberra, Australia) were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Scoresby, VIC, Australia) supplemented with 2 mM L-glutamine, 10% Serum Supreme (Lonza, Tullamarine, VIC, Australia), 100 units/ml interleukin-3 (IL-3). Cells were starved by removing IL-3 or GH and reducing serum to 0.5% by washing in phosphate-buffered saline (PBS) and culturing in fresh media as above except containing 0.5% foetal bovine serum and no IL-3.

HEK293 (ATCC CRL-1573), Plat-E (kindly provided by Professor T Kitamura, Tokyo University, Tokyo, Japan),<sup>61</sup> and 293T (ATCC CRL-3216) cell lines were cultured in Dulbecco's modified Eagle's medium with 10% foetal bovine serum. HEK293 cells were starved by washing in PBS and culturing in Dulbecco's modified Eagle's medium with 0.5% foetal bovine serum for 16 h. BEAS-2B (Cell Bank Australia, Westmead, NSW, Australia; 95102433) cells were maintained in complete LHC-9 medium (Gibco, Thermo Fisher Scientific). Flasks were precoated with 0.01 mg/ml fibronectin (Becton Dickinson, North Ryde, NSW, Australia), 0.03 mg/ml collagen (Sigma-Aldrich, St Louis, MO, USA) and 1  $\mu$ g/ml bovine serum albumin as described by Cell Bank Australia. BEAS-2B cells were starved in RPMI-1640 supplemented with 2 mM L-glutamine and 0.5% bovine serum albumin medium overnight. All cells were maintained in 5% CO<sub>2</sub> incubator at 37 °C. All cell lines were routinely tested for mycoplasma and confirmed as negative.

Transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Inhibitors BFA, DAPT, MG-132 (Z-Leu-Leu-Leu-al), clasto-Lactacystin  $\beta$ -lactone were obtained from Sigma-Aldrich. hGH was expressed and purified as described.<sup>62</sup>

### Retroviral transduction

Ba/F3 cells were transduced to stably express WT or GHRP495T by ecotropic retroviruses produced by transfection of Plat-E cells with GHR genes cloned in pMX-GW-IRES-Puro essentially as described.<sup>57</sup> Cells were selected with puromycin and total GHR levels were determined by immunoblotting against N-terminal HA-tag (clone161B2, Covance, Princeton, NJ, USA). Cells were sorted by fluorescence-activated cell sorting for similar levels of surface GHR by the HA-tag as described.<sup>29</sup>

BEAS-2B and HEK293 were transduced to express WT or mutant GHR by pantropic retroviruses produced by transfection of 293T cells with GHR genes cloned in pQCXP CMV/TO DEST<sup>58</sup> and retroviral packaging vectors pVPack-GP and pVPack-VSV-G (Stratagene, La Jolla, CA, USA) with Lipofectamine 2000 and virus was harvested according to the manufacturer's instructions (Stratagene). Transduced cells were selected by puromycin and GHR expression was determined by immunoblotting.

### Quantitative RT-PCR

HEK293 and BEAS-2B transduced cell lines were stimulated with GH as indicated, and RNA extracted with TRIzol. Similarly, murine hepatic tissue was homogenised in TRIzol (Life Technologies, Mulgrave, VIC, Australia) and RNA was extracted as per the manufacturer's guidelines. Complementary DNA was synthesised using iScript RT Supermix (Bio-Rad, Gladesville, NSW, Australia) and transcripts levels quantified using Sybr Green Mix (Thermo Fisher Scientific) on a ViiA7 machine (Applied Biosystems, Thermo Fisher Scientific). Analysis was performed by calculating the change in Ct value between the gene of interest against the housekeeping gene,  $\beta$ -2 microglobulin (*B2M*) or *GAPDH* and represented as relative levels. Primers used are listed in Supplementary Table 1.

### Proliferation assay

Ba/F3 cells transduced with WT and GHRP495T were starved overnight and seeded at a cell density of  $1 \times 10^4$  cells/ml duplicate in growth media supplemented with GH at 4.5 nM instead of IL-3 (day 0). Cells were counted daily by Trypan blue exclusion using a hemocytometer. Proliferation in BEAS-2B was measured by BrdU incorporation. Briefly, cells were grown on coverslips at equal seeding densities and serum starved the following day to induce cell cycle synchronisation. Cells were then treated with GH at 4.5 nM for 24 and 48 h. Before fixing, cells were given 20  $\mu$ M 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) for 1 h. Cells were washed with PBS



and fixed in 70% ethanol and coverslips were stained with BrdU antibody (#5292, Cell Signaling, Danvers, MA, USA) as per the manufacturer's guidelines, followed by Alexa Fluor-488 antibody and counterstained with 10 µg/ml 4,6-diamidino-2-phenylindole (DAPI). Random fields of views were first imaged for DAPI and the same field of view was imaged for BrdU and quantified blind. Data are represented as percentage of BrdU-positive cells.

### GH signalling analysis and western blotting

Cells were serum-starved overnight and stimulated with 2.3 nM GH in starve medium for 15 min. GH was removed by two PBS washes and cells were maintained in starve medium at 37 °C until harvested (by scraping for adherent cell lines) at subsequent time points as described in the results section. For BFA experiments, starved cells were pre-treated with 1 µg/ml BFA for 1 h before GH treatment and BFA was maintained when media was replaced. At subsequent time points, cells were washed in cold PBS and subjected to protein extraction in cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5; 0.5% sodium dodecyl sulphate (SDS), 1% NP-40, sodium deoxycholate supplemented with protease inhibitors (Roche, Sydney, NSW, Australia, EDTA-free), 10 mM NaF, 1 mM Na<sub>4</sub>O<sub>7</sub>P<sub>2</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>) as described previously.<sup>57</sup> Hepatic tissue from WT and *ghr*<sup>-/-</sup> mice<sup>63</sup> were homogenised in cold RIPA buffer. Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific). Equal amount of protein was boiled in sample buffer (15 mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 10 mM DTT) at for 5 min and resolved on SDS–polyacrylamide gel electrophoresis gels with transfer to polyvinylidene difluoride and immunoblotted as described previously using P-STAT5 (Tyr694/Tyr699) (#4322, Cell Signaling), STAT5 (#sc-835, Santa Cruz Biotechnology, Dallas, TX, USA), P-AKT (Thr308) (#13038, Cell Signaling), P-AKT (Ser473) (#4060, Cell Signaling), AKT (#9272, Cell Signaling), GAPDH (#2118, Cell Signaling), β-TUBULIN (#2128, Cell Signaling), P-STAT3 (Tyr705) (#9145, Cell Signaling) and STAT3 (#4904, Cell Signaling).<sup>57</sup>

### Co-immunoprecipitation

Transduced HEK293 cells were transfected with 2 µg of CISH or SOCS2 expression plasmid for 24 h. Cells were starved overnight before GH stimulation (2.3 nM) for 15 min. Cells were harvested in immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 0.5% Triton X-100 and supplemented with 100 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, complete EDTA-free protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were equalised based on total protein (determined by BCA assay) then first pre-cleared and subsequently subjected to CISH (#8731, Cell Signaling) or SOCS2 antibody (#2779, Cell Signaling) pull down for 2 h at 4 °C on a rotating wheel and Protein A/G sepharose beads (GE Life Science, Parramatta, NSW, Australia) were added for another 2 h at 4 °C. Beads were washed in the immunoprecipitation buffer for five times and boiled in sample buffer (15 mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 10 mM DTT) for 5 min. The supernatant was resolved by SDS–polyacrylamide gel electrophoresis.

### Total internal reflection fluorescence microscopy

HEK293 cells transduced with WT GHR or GHRP495T (GFP-tagged) were seeded on glass-bottom culture dishes (MatTek Corporation, Ashland, MA, USA) and transfected with SOCS2-mCherry 24 h before visualisation on a TIRF microscope (Marianas, SDC Everest™, Intelligent Imaging Innovations Inc., Denver, CO, USA) fitted with a 100x oil immersion objective (NA = 1.46, Carl Zeiss, Jena, Germany) using EMCCD cameras (QuantEM 512sc) and Slidebook software version 5.5 (3i Inc., Denver, CO, USA). Cells were imaged in live-cell imaging medium (Thermo Fisher Scientific) and analysis performed as previously described.<sup>64</sup>

### Nuclear magnetic resonance

WT residues GHR-ICD<sub>1455-5588</sub>/GHR-ICD<sub>1436-5570</sub> (numbers ± signal peptide) and with the variant P495T (or P477T) GHR-ICD<sub>1455-5588</sub><sup>P495T</sup> were cloned into the pGEX-4T1 vector as a GST fusion separated by a TEV-cleavage site. The recombinant proteins were expressed in *Escherichia coli* BL21(DE3) in <sup>15</sup>N,<sup>13</sup>C minimal media and purified as described.<sup>34</sup> Backbone chemical shifts were assigned from similar sets of nuclear magnetic resonance spectra as in Haxholm *et al.*<sup>34</sup> for both proteins in 20 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> and in 8 M urea at pH 7.3, respectively. Samples of 350 µl were added 10% (v/v) <sup>2</sup>H<sub>2</sub>O, 5–8 mM tris(2-carboxyethyl)phosphine and 0.5 mM 2,2-dimethyl-2-silapentane-5-sulphonic acid for referencing. Transient

secondary structures were identified from secondary chemical shifts calculated by subtracting the δC<sup>α</sup> for each residue in 7–8 M urea from those in 20 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>. The population of transient α-helices (THs) was assessed as previously described.<sup>65</sup>

### Bioinformatics analysis

Multiple sequence alignment of GHR was carried out on protein sequences sourced from NCBI database using Clustal Omega tool (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).<sup>66</sup>

Microarray expression data were from the NCBI GEO DataSet database (<http://www.ncbi.nlm.nih.gov/gds/>).<sup>67</sup> Array profiling for GHR levels was performed on 18 SCC and 40 lung adenocarcinoma tissues data set (GSE10245)<sup>24</sup> and represented as dot plot. Individual data points for relative expression of numerous genes expressed in paired clinical samples representing 60 normal lung tissue and 60 NSCLC tumours derived from non-smoking females (GSE19804)<sup>25</sup> was performed and plotted as mean ± s.e.m. Data for NSCLC shown are only for female cohorts as no GeoDATA set was available for NSCLC for the male sub-population. This analysis was carried out to indicate the increase in GHR and decrease in SOCS2 levels in the available NSCLC cohort. Data were analysed using Student's *t*-test.

### Statistical analysis

Densitometric quantitation was performed on immunoblots using ImageJ software (NIH, Bethesda, MD, USA). Values were normalised to β-TUBULIN or GAPDH and represented as arbitrary units. Statistical analyses were performed using GraphPad-PRISM software (La Jolla, CA, USA) based on a minimum of three independent experiments using Student's *t*-test for comparison between two groups or one-way analysis of variance (ANOVA) test followed by a Tukey's post-test for analysing means at one variable or two-way ANOVA followed by a Bonferroni post-test for analysing means at two independent variables. Significance was scored as: \*\*\*\**P* < 0.0001; \*\*\**P* < 0.001; \*\**P* < 0.01; \**P* < 0.05.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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